Supporting Information for

In situ fluorescent profiling of living cell membrane proteins at single-molecule level

Yuanyuan Fan, Lu Li,* Meng Lu, Haibin Si, and Bo Tang*

College of Chemistry, Chemical Engineering and Materials Science, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Institute of Molecular and Nano Science, Shandong Normal University, Jinan 250014, P. R. China

Author Email: tangb@sdnu.edu.cn, lilu5252@163.com
Fax: 86-531-86180017
Table of contents

1. Experimental Section .................................................................S3
2. Sequences of the used oligonucleotide probes.................................S5
3. The verification of the high selective detection for membrane protein PTK7......S6
4. The condition optimization of ligation process........................................S7
5. The condition optimization of amplification process...............................S8
6. The amplification length determined by fluorimetric pyrophosphate (PPi) ..........S9
7. The cell toxicity of in situ ligation and amplification process.........................S10
8. Confocal fluorescence images of Hela cells incubated with fluorescent amplified products at 37 °C for different time.............................................................S11
9. Confocal fluorescence images and linear intensity analysis of Hela cells incubated with fluorescent amplified products at 4 °C for different time......................S12
10. Confocal fluorescence images before and after deconvolution processing........S13
11. Confocal fluorescence images of RCA products..................................S14
12. The dimensions of single fluorescence spots on cell membrane....................S15
13. Flow cytometry plot of three different cell lines with different expression level of PTK7.................................................................................................................S16
14. Confocal fluorescence images of EpCAM on MCF-7 cell surface labeled by HP2-Cy5 during EMT.................................................................S17
1. Experimental Section

Reagents and materials. Oligonucleotides, deoxynucleotides (dNTPs) and TE buffer were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). T4 DNA ligase was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Phi29 DNA polymerase, exonuclease I and exonuclease III were obtained from New England Biolabs (Ipswich, MA). TGF-β1 were purchased from R&D Systems ((Minneapolis, MN, U.S.A.) Purified water was obtained from Wahaha (Hangzhou Wahaha Group Co., Ltd.) All chemicals and solvents used were of analytical grade. The sequences of the oligonucleotides were listed in Table S1.

Gel electrophoresis was performed on a DYCYZ-24DN Electrophoresis Cell (LIUYI, Beijing, China) and GelDoc-It Imaging Systems (UVP, Cambridge, U. K.). The fluorescent spectra were measured using a Cary Eclipse Fluorescence Spectrophotometer (Varian, CA). In the MTT assay, a microplate reader (RT 6000, Rayto, U.S.A.) was used to measure the absorbance. Confocal fluorescence imaging was conducted with a TCS SP8 confocal laser scanning microscope (Leica Co., Ltd. Germany). Flow cytometry data were carried on an ImageStream MarkII (Merck Millipore, Seattle, U. S. A.) imaging cytometer.

Cell culture and extraction. Hela, CCRF-CEM, A549, MCF-7 cell lines were all obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM (BI) plus 10% fetal bovine serum (BI) and 1% penicillin−streptomycin (BI) and maintained at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air. The erythrocyte and hepatocyte were obtained from the mice cardiac tissue and liver tissue by gentle grinding with a 40 μm cell strainer.

RCA reaction. Before ligation, 30 μM Hair Probes (HP, contained primer sequence for circular template ligation) were firstly hybridized with 30 μM Circle Probes (CP) in 10 μL TE buffer, then heated to 95 °C for 3 min and cooled to room temperature over a 15 min period. Ligations were performed in a total of 20 μL reaction solution, which contained 10 μL hybridized product, 400 U T4 DNA ligase and 1 × ligation buffer (10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 μM ATP). The reactions were incubated at room temperature for 16 h and were terminated by heating at 80 °C for 15 min. Adding 10 U exonuclease I and 100 U exonuclease III at 37 °C overnight to remove the unreacted HP and CP.

Next, the ligation products were added to the RCA reaction mixture containing 1× phi29 reaction buffer (40 mM Tris-HCl pH 7.5, 50mM KCl, 10 mM MgCl₂, 5 mM (NH₄)₂SO₄, and 4 mM DTT), 1×BSA, 5 units phi29 DNA polymerase and 400 μM dNTPs. The reactions were incubated at 30 °C for 6 h and were terminated by heating at 65 °C for 10 min.

At last, the amplified products were incubated with 100 μM Cy5-DNA at 37 °C for 1 h. The hybridized products could be performed to the subsequent fluorescence spectra measurement and fluorescence imaging.

Gel electrophoresis. A total of 10 M DNA samples mixed with loading buffer and 6 μL DNA marker were added into the sample holes of 1.5 % W/V agarose. Then agarose gel electrophoresis assay was conducted at 100 V for 80 min and the running buffer was 1×TBE. After electrophoresis, oligonucleotides were stained by EB and visualized by a GelDoc-It Imaging Systems.

Fluorescence spectra measurement. The RCA products were added into the cells (5×10⁶ cells/mL) and reacted at 4 °C for 1 h. After centrifugation (800 r/min, 5 min) for three times, the fluorescence of Cy5 was collected from 655 and 710 nm by the use of maximal excitation wavelength at 649 nm.
Flow cytometry. Firstly, the fluorescent RCA products were incubated with the cells (5×10^5 cells/mL) at 4 °C for 1 h. Amnis ImageStream MarkII imaging cytometer with 4-laser attachment (375, 488, 561 and 642) were used to acquire cells fluorescent intensity. 5,000 events were obtained at 40 × magnification. We used 642 nm wavelength laser to excite Cy5 fluorophore and collected the fluorescence by the 642-745 nm spectral detection channels.

Laser confocal fluorescence imaging of PTK7. The cells were incubated with the RCA products for 1 h and then washed three times with PBS to remove the excess probes. Finally, the high-resolution fluorescent images were acquired with a TCS SP8 confocal laser scanning microscope using a 633 nm excitation source and deconvolution processing system which was equipped on the confocal imaging system.
2. Sequences of the used oligonucleotide probes.

Table S1. The bold sequences could hybridize to each other by complementary base pairing. The sequences with underline are the same with Cy5-DNA.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair Probe</td>
<td>ATCTAACTGCTGCGCCGCCCCGGAAAAATACGGTTAGAT <strong>TGTATAGATGGTAGTA</strong></td>
</tr>
<tr>
<td>Circular Probe</td>
<td>P-</td>
</tr>
<tr>
<td></td>
<td><strong>TCTATACAA</strong>TACCACTCCATCATCCAAACATATCAACG</td>
</tr>
<tr>
<td></td>
<td>CTACCACTCCATCATCCAAACATATCAACGCTACCACTC</td>
</tr>
<tr>
<td></td>
<td><strong>CTACTACCA</strong></td>
</tr>
<tr>
<td>Cy5-DNA</td>
<td>Cy5-CGCAACTATATACCAACCTACTAC</td>
</tr>
<tr>
<td>HP-2</td>
<td>CAC TAC AGA GGT TGC GTC TGT CCC ACG TTG TCA</td>
</tr>
<tr>
<td></td>
<td>TGG GGG GTT GGC CTG <strong>TTGTATAGATGGTAGTA</strong></td>
</tr>
</tbody>
</table>
3. The verification of the high selective detection for membrane protein PTK7.

**Figure S1.** Fluorescence intensity of different cell lines after recognition with hairpin probe and signal amplification by RCA.
4. The condition optimization of ligation process.

**Figure S2.** The optimal ligation time (A), temperature (B) and concentration of T4 DNA ligase (C).
5. The condition optimization of amplification process.

**Figure S3.** The optimal amplification time (A), temperature (B) and concentration of phi29 DNA polymerase (C), and the optimal concentration of dNTPs (D).
6. The amplification product length determined by fluorimetric pyrophosphate (PPi) assay.

Table S2. The amplification product length at different amplification time.

<table>
<thead>
<tr>
<th>Time</th>
<th>C_{PPi}/μM</th>
<th>DNA length per amplicon/nt</th>
<th>Tem. Repeats per amplicon/copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td>17.19</td>
<td>42975</td>
<td>511</td>
</tr>
<tr>
<td>40 min</td>
<td>24.75</td>
<td>61875</td>
<td>736</td>
</tr>
<tr>
<td>1 h</td>
<td>25.95</td>
<td>64875</td>
<td>772</td>
</tr>
<tr>
<td>2 h</td>
<td>30.00</td>
<td>75000</td>
<td>892</td>
</tr>
</tbody>
</table>

Table S3. The amplification product length at different concentration of dNTPs.

<table>
<thead>
<tr>
<th>dNTPs</th>
<th>C_{PPi}/μM</th>
<th>DNA length per amplicon/nt</th>
<th>Tem. Repeats per amplicon/copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 nM</td>
<td>2.132</td>
<td>5330</td>
<td>63</td>
</tr>
<tr>
<td>10 nM</td>
<td>2.486</td>
<td>6215</td>
<td>74</td>
</tr>
<tr>
<td>100 nM</td>
<td>3.985</td>
<td>9963</td>
<td>119</td>
</tr>
<tr>
<td>10 μM</td>
<td>6.116</td>
<td>15290</td>
<td>182</td>
</tr>
<tr>
<td>500μM</td>
<td>16.65</td>
<td>41625</td>
<td>496</td>
</tr>
<tr>
<td>1 mM</td>
<td>20.90</td>
<td>52250</td>
<td>622</td>
</tr>
</tbody>
</table>
7. The cell toxicity of \textit{in situ} ligation and amplification process.

\textbf{Figure S4.} The evaluation of labeling and amplification of membrane proteins on living cells. (A) Cell viability with different process. Group 1, the control experiment; Group 2, in situ ligation; Group 3, ligation in solution and then performed in situ amplification process; Group 4, ligation and amplification process in solution firstly and then adding the amplified products into cell suspension to co-incubation at 37 °C; Group 5, adding the amplified products into cell suspension to co-incubation at 4 °C.
8. Confocal fluorescence images of Hela cells incubated with fluorescent amplified products at 37 °C for different time.

Figure S5. Confocal fluorescence images of Hela cells incubated with fluorescent amplified products at 37 °C for different time.
9. Confocal fluorescence images and linear intensity analysis of Hela cells incubated with fluorescent amplified products at 4 °C for different time.

Figure S6. (A). Confocal fluorescence images of the cells incubated with fluorescent RCA products at 4 °C for different time. (Scale bar: 50 μm) (B). Linear intensity analysis of single cell in confocal fluorescence images (A)
10. Confocal fluorescence images before and after deconvolution processing.

Figure S7. Confocal fluorescence images of Hela cells incubated with fluorescent amplified products before (top) and after deconvolution processing (down).
11. Confocal fluorescence images of RCA products.

Figure S8. Confocal fluorescence images of RCA products generated in solution and deposited on glass slides. The image on the right is a larger version of the rectangular area in the left image. Most of the RCA products are with diameters of approximately 0.5 μm, which are similar to the single fluorescence spots on cell membrane (Fig. S9). Therefore, the single fluorescence spots on cell membrane are come from single RCA products. In addition, one membrane protein can combine to only one RCA products by specific aptamers, which proves that the visualization analysis of membrane proteins was at a single-molecular level. The background may come from the unreacted Cy5-DNA.
12. The dimensions of single fluorescence spots on cell membrane.

**Figure S9.** The dimensions analysis of representative fluorescence spots on cell membrane in Fig. 3 and 4 (leftmost images). Most of the spots are with diameters of approximately 0.5 μm, which are similar to those in Fig. S8. (Scale bar: 2 μm)
13. Flow cytometry plot of three different cell lines with different expression level of PTK7.

**Figure S10.** Flow cytometry plot of three cells with different expression level of PTK7.
14. Confocal fluorescence images of EpCAM on MCF-7 cell surface labeled by HP2-Cy5 during EMT.

Figure S11. Confocal fluorescence images of EpCAM on MCF-7 cell surface labeled by HP2-Cy5 during EMT.